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Mechanism of carnitine transport catalyzed by carnitine carrier from rat brain mitochondria

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Abstract. The transport mechanism of reconstituted carnitine carrier purified from rat brain mitochondria was studied kinetically. Short and medium chain acyl carnitine derivatives had much higher affinity to the carnitine carrier in comparison with long chain acyl carnitine derivatives, therefore both homologous (carnitine/carnitine) and heterologous (carnitine/acetylcarnitine) antiports were analysed. A complete set of half-saturation constants was establiblished for various substrate concentrations on both the external and the internal side of the membrane. Bisubstrate initial velocity analyses of the exchange reaction resulted in a kinetic pattern which is consistent with a sequential antiport mechanism. This type of mechanism implies formation of a ternary complex of the carrier with one internal and one external substrate molecule before the transport reaction occurs.

Key words: carnitine carrier, transport mechanism (brain, mitochondria)

INTRODUCTION

Transport of ions or nonionized metabolites through biological membranes may occur by one of three alternative mechanisms: uniport, symport or antiport. The latter represents a situation in which one molecule of the substrate is transported across the membrane in exchange for another, either identical or different, molecule from the opposite side. The process of transfer occurs due to conformational changes of the transporting proteins and, according to the theory of Läuger, by fluctuations of energy barriers (Läuger 1984).

In case of a carrier, whose name originates from mobile antibiotic ionophores, a ping-pong mechanism has been postulated, i.e., a situation where binding of a substrate on one side of the membrane lowers the energy barrier, promoting transfer of the substrate followed by binding of another substrate molecule on the other side of the membrane. According to this mechanism, only one substrate molecule at a time would be bound to the transporting protein. An alternative, sequential mechanism stipulates a situation where two binding sites (for two different substrates) have to be occupied before transport takes place; this implies lowering energy barriers on both sides of the membrane.

Mitochondria, separated from the cytoplasm by two membranes, contain several specialized systems which allow the net transport and/or the exchange of metabolic substrates between the matrix and the cytoplasm (LaNoue and Schoolwerth 1979). A ping-pong mechanism has been postulated for the mitochondrial carriers when the process of exchange was studied with intact mitochondria, as described for ADP/ATP (Klingenberg 1985) and aspartate/glutamate (LaNoue et al. 1979a) carriers. However, studies on the oxoglutarate carrier (Sluse et al. 1979) and the ADP/ATP carrier (Duyckaerts et al. 1980) gave arguments in favour of a sequential type of mechanism for binding and transport.

Recently, many of these carriers have been extracted from the membrane and obtained in a pure and reconstitutively active form (Palmieri et al. 1993). Kinetic studies performed in a reconstituted system allow a much more precise control of the substrate concentration in the inner (inside vesicles) compartment. The results of two-reactant initial velocity studies of the exchange reaction of the reconstituted aspartate/glutamate (Dierks et al. 1988), 2-oxoglutarate (Indiveri et al. 1991), tricarboxylate (Bisaccia et al. 1993), dicarboxylate (Indiveri et al. 1993) and pyruvate (Nałęcz 1994) carriers point to a sequential type of mechanism as a basis of the translocating activity performed by carriers of the inner mitochondrial membrane.

We have recently purified the carnitine/acylcarnitine translocase from rat brain mitochondria (Kamińska et al. 1993). The carrier was found to consist of a polypeptide with the molecular mass of 33 kDa, i.e., very similar to that described for liver (Indiveri et al. 1990).

Recently a kinetic analysis of the carnitine carrier from liver has been reported (Indiveri et al. 1994) and it was demonstrated that this protein in the reconstituted system followed a ping-pong mechanism. Due to the observed differences in substrate specifity between carnitine carrier from brain (Kamińska et al. 1993) and liver (Indiveri et al. 1991), the present study was undertaken. The two-substrate kinetic analysis was applied to the reconstituted carnitine carrier isolated from rat brain mitochondria. The results imply that the carnitine homoexchange as well as the exchange with acetylcarnitine occurs by a sequential mechanism and that the ternary complex must be formed during this reaction.

METHODS

Materials

L-[methyl-^{3}H]carnitine was purchased from Amersham. L-Acetylcarnitine, L-propionylcarnitine and L-palmitoylcarnitine were delivered by Serva. DL-hexanoylcarnitine, DL-octanoylcarnitine, DL-lauroylcarnitine, DL-stearoylcarnitine, cardiolipin, Triton X-100 and Pipes were from Sigma, N-ethylmaleimide from Merck. Hydroxyapatite (Bio-Gel HTP) was from Bio-Rad. L-Car-

nitine, Amberlite XAD-2 and L-sn-phosphatidylcholine from egg yolk were provided by Fluka and Sephadex G-50 by Pharmacia. Ultima Gold (Packard) was used as a scintillation cocktail. All other reagents were of analytical grade.

Three-month old male albino WAG rats were used throughout this study. Brain mitochondria were prepared essentially as described by Booth and Clark (1978).

Purification and reconstitution of the carnitine carrier.

The carnitine carrier was purified from rat brain mitochondria as described elsewhere (Kamińska et al. 1993). The partially purified preparation either of HTP eluate or of the third celite fraction was used in all experiments.

For reconstitution, egg yolk phosphatidylcholine liposomes were prepared by sonication of 100 mg of phospholipid, in 1 ml of the medium containing 20 mM NaCl, 1 mM EDTA, 10 mM Pipes (pH 7.0) and carnitine or acetylcarnitine at concentration indicated in the figure legends.

Aliquots of 500 µl containing 5 µg protein of celite eluate were mixed with 120 µl 10 % Triton X-100, 200 l liposomes, 1.2 mg cardiolipin and carnitine or acetylcarnitine in 50 mM sodium phosphate buffer, pH 7.0. Triton X-100 was removed by incubation of this mixture with 2 g Amberlite XAD-2 beads for 5 h at 20°C ("batch" reconstitution). Proteoliposomes were passed through Sephadex G-50 column preequlibrated with 60 mM NaCl, 10 mM Pipes (pH 7.0).

Measurement of carnitine carrier activity

The activity of the carnitine carrier was measured by $carnitine_{ex}/carnitine_{in}$ exchange or $carnitine_{ex}/acetylcarnitine_{in}$ exchange.

In order to measure this activity in the reconstituted system, proteoliposomes were incubated for the indicated time at 30°C with L-[methyl-^{3}H]carnitine (0.5-5.0 Ci/mol). The inhibitor stop method with 1 mM NEM (Palmieri and Klingenberg 1979) was applied in order to terminate the reaction. External carnitine was removed by passing of aliquots of the assay mixture through Sephadex G-50 columns (1.0 x 14.0 cm) preequilibrated and washed with 50 mM NaCl. Samples corresponding to the void volume were collected into scintillation vials for radioactivity counting.

The values reported represent total accumulation of radioactive L-[methyl-^{3}H]carnitine after subtraction of the background ("leak" and adsorption) measured with liposomes incubated in the presence of carnitine for the given time (this control never exceeded 10% of the "active" accumulation).

Other procedures

Protein content was determined by the Lowry procedure (Lowry et al. 1951), modified by the addition of 1% SDS (Nałęcz et al. 1986).

RESULTS AND DISCUSSION

The carnitine carrier from the inner mitochondrial membrane is capable of exchanging carnitine for its acyl derivatives of various chain length (Murthy and Pande 1984). In a previous report (Kamińska et al. 1993) we have shown that short and medium chain acyl derivatives, namely acetyl- and propionylcarnitine, were more potent inhibitors of carnitine/carnitine exchange than palmitoylcarnitine. In order to clarify whether the reaction catalyzed by the carnitine carrier was dependent on the chain length of the acyl moiety in a stepwise or proportional manner, we have tested seven esters of carnitine. The addition of acetylcarnitine was found to decrease the carnitine/carnitine exchange by 80% (Fig. 1). With the increase in the number of carbon atoms in the acyl chain of carnitine derivatives the inhibitory effect was less and less pronounced, decreasing the rate in the presence of stearoylcarnitine to only 37% of the control value. Figure 1 shows that the inhibition of carnitine/carnitine exchange with respect to the number of carbon atoms in the acyl chain of acylcarnitines could be expressed as a linear dependence with the correlation coefficient r^2=0.958.

The affinity for carnitine transport on the external membrane surface of the reconstituted carnitine

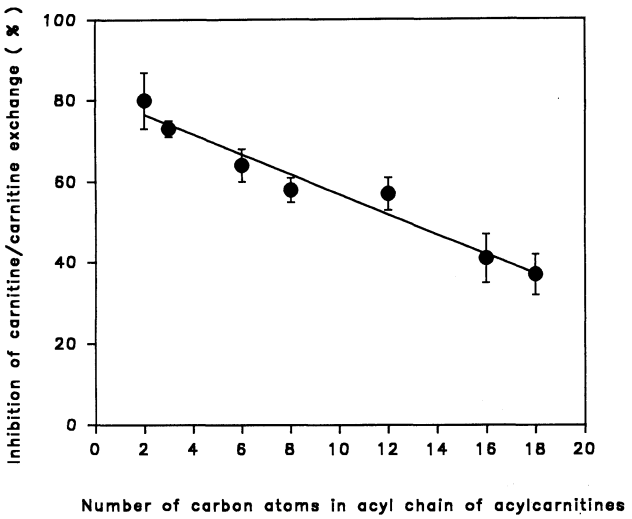

Fig. 1. Inhibition of the carnitine transport by acylcarnitine derivatives. The carnitine carrier was isolated from rat brain mitochondria. Proteoliposomes were preloaded with 20 mM carnitine. Transport was measured in reconstituted system after 7 min as accumulation of radioactive 0.5 mM carnitine in the presence of the following acylcarnitine derivatives 3 mM: acetylcarnitine, propionylcarnitine, hexanoylcarnitine, octanoylcarnitine, lauroylcarnitine, palmitoylcarnitine, stearoylcarnitine. Data are the means ±SD for three experiments. The control value in the absence of acylcarnitine derivatives equaled 22.8±1.4 μmol/min per g protein.

carrier was determined by the forward exchange method. The K_m value for carnitine at the external side of the liposomal membrane was 0.54±0.06 mM in the presence of 20 mM internal carnitine and external carnitine ranging from 0.2 mM to 5 mM (Fig. 2). This K_m value is in the same range as the values obtained for the reconstituted carrier from liver (0.51 mM) (Indiveri et al. 1994) and in intact mitochondria (0.3 mM - 2 mM) (Pande and Parvin 1978, Pande and Parvin 1979, Idell-Wenger 1981).

It was shown previously that the time course of carnitine accumulation in the exchange reaction (concentration of carnitine was 20 mM internal and 0.5 mM external) catalyzed by the reconstituted carnitine carrier was still linear up to at least 10 min of incubation (Kamińska et al. 1993). Hence, the rate of this reaction after 5 min of incubation was considered "initial" and taken as such for further kinetic analysis. Additional support for such an experimental setup came from the fact that no saturation of accumulation process was observed even after 30 min of incubation (not shown). Similar data were obtained for various concentrations of internal and external carnitine (Fig. 3A and B). It is also worth pointing out that the linear slopes presented in Fig. 3 do not extrapolate to zero carnitine accumulation at zero time of incubation. This most likely reflects adsorption of carnitine to proteoliposomes that is concentration-dependent.

Figure 4 shows the results of two-reactant initial--velocity study (two-substrate analysis) of the homoexchange reaction of internal and external carnitine. External carnitine was varied from 0.05 to 0.5 mM and internal from 2.0 to 20.0 mM. As already mentioned the antiport velocities were calculated from the carnitine uptake measured 5 min. When the kinetic data were analysed by reciprocal plots (Fig. 4A and B) straight lines intersecting at a common point were obtained. This pattern, in contrast to a set of parallel lines which is expected in the case of a ping-pong reaction mechanism, is indicative of a sequential type of mechanism (Cleland 1970). The characteristic feature of this mechanism is the formation of a ternary complex of the carrier protein

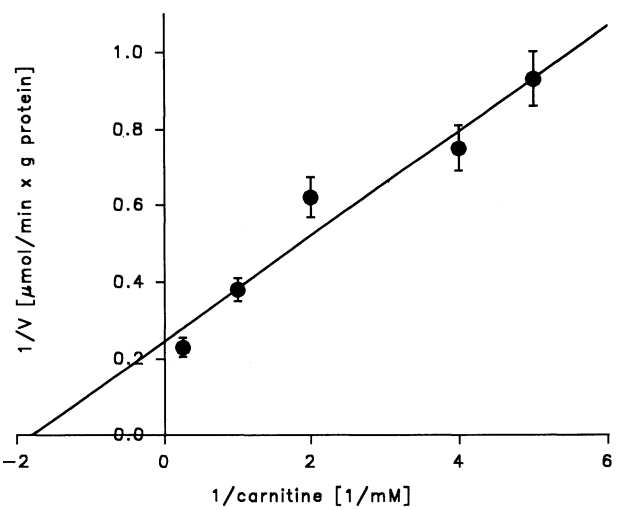

Fig. 2. Lineweaver-Burk plot for forward-exchange (carnitine/carnitine) of the reconstituted carnitine carrier from rat brain mitochondria. The measurements were performed under variation of external carnitine (0.2, 0.25, 0.5, 1.0 and 5.0 mM). The concentration of internal carnitine was 20 mM. Transport was measured after 5 min as accumulation of radioactive carnitine. Data are the means ±SD for five experiments.

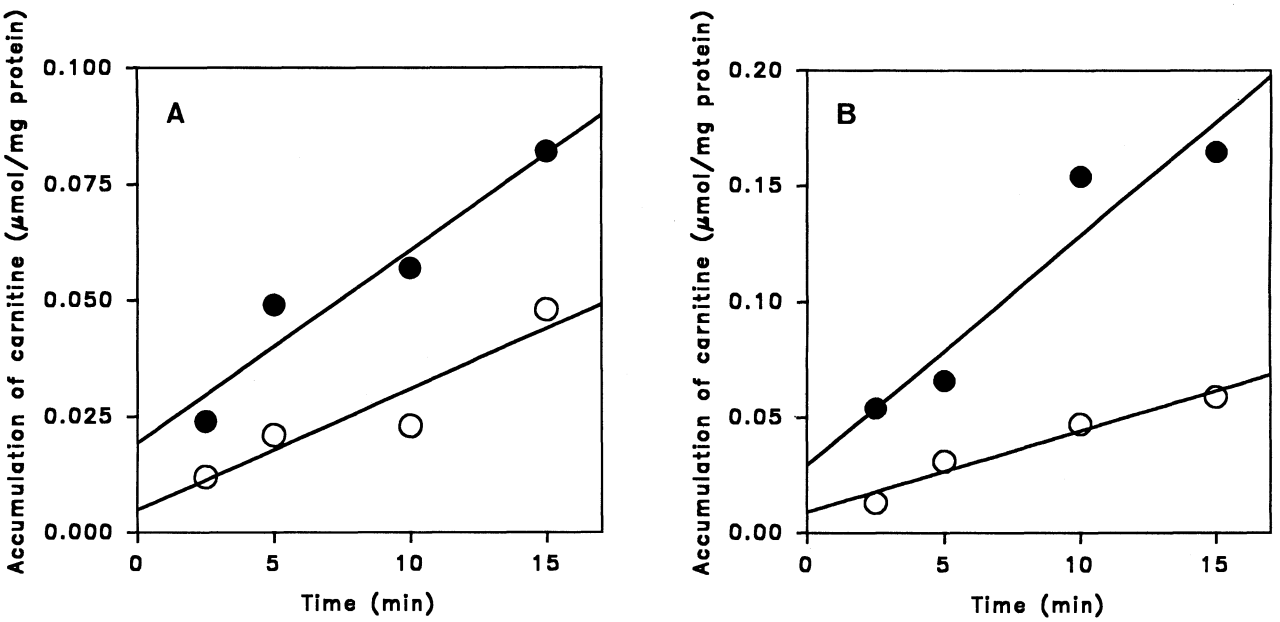

Fig. 3. The time course of carnitine$_{in}$/carnitine$_{ex}$ exchange activity catalyzed by the reconstituted carnitine carrier from rat brain mitochondria. The transport was measured as accumulation of radioactive carnitine in proteoliposomes after indicated time, presented here as total values without subtraction of "leak" and adsorption of carnitine (see Methods). Concentrations of external carnitine were: 0.05 mM (open circles) and 0.5 mM (filled circles). Concentrations of internal carnitine were: 2 mM (A) and 20 mM (B).

with two substrate molecules, prior to translocation phenomena. The data were further analysed in secondary plots of the slopes and the ordinate intercepts of the primary curves vs. the reciprocal concentration of the so-called non-varied substrate (Fig. 4C and D refer to the data of Fig. 4A and B, respectively). From the intercept of the secondary plots, K_m values could be derived when extrapolated to infinite concentration of the respective second substrate (Cleland 1970). For carnitine/carnitine exchange the apparent K_m for internal carnitine was 8.12±0.72 mM and the corresponding value for external carnitine was 0.42±0.03 mM.

Two-substrate analysis of carnitine/acetylcarnitine heteroexchange (Fig. 5) led to very similar kinetic patterns as obtained for the homoexchange reaction. Also in these cases an intersection point located close to the abscissa was observed, demonstrating that homo- and heteroexchange followed the same basic mechanism. For carnitine/acetylcarnitine exchange the apparent K_m for internal acetylcarnitine was 7.12±0.45 mM, whilst for external carnitine was 0.19±0.02 mM.

The fact that K_m values for external and internal substrates were so different suggests an asymmetric incorporation of the carrier molecule into the artificial lipid membrane. In both cases the affinity of the transporting protein was very high towards external carnitine and much lower towards the internal substrate, which suggests that not only does the reconstitution not occur randomly, but that the protein *per se* is functionally asymmetric. This has also been found in mitochondria, where the exchange of externally added carnitine with the internal carnitine derivative(s) is favored over the reversed exchange, i.e., mitochondria express the same carrier polarity as observed here for the reconstituted system.

Preferential right-side-out orientation after reconstitution has already been reported for the carnitine carrier from rat liver mitochondria (Indiveri et al. 1994) and for some other mitochondrial carriers, e.g., the phosphate carrier (Stappen and Krämer 1993), the dicarboxylate carrier (Indiveri et al. 1993), the tricarboxylate carrier (Bisaccia et al. 1993) and the aspartate/glutamate carrier (Dierks and Krämer 1988).

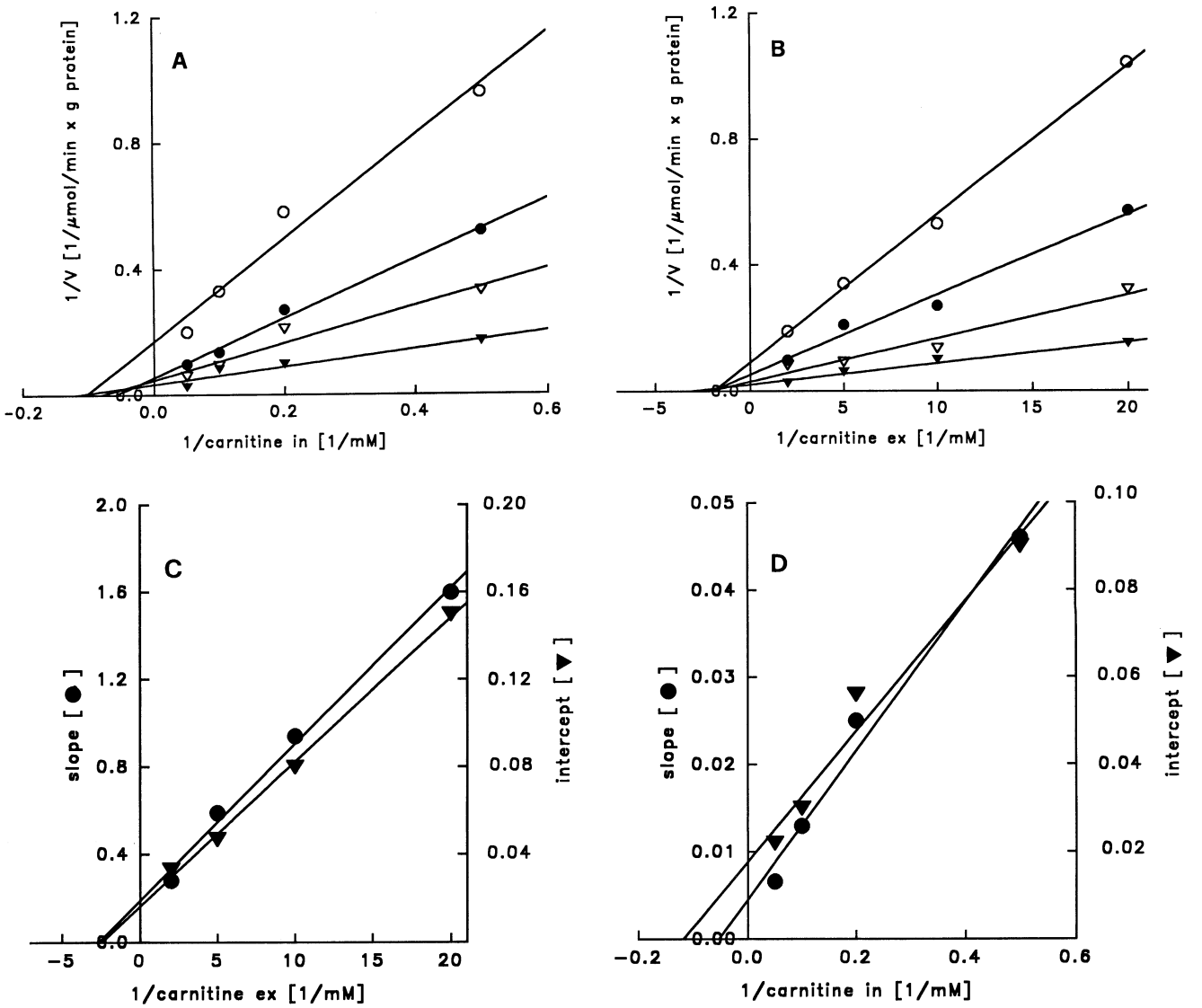

Fig. 4. Two-substrate analysis of the carnitine/carnitine homologous exchange reaction mediated by the reconstituted carnitine carrier. The transport was measured as accumulation of radioactive carnitine after 5 min as described in Methods. A, B, Lineweaver-Burk plots showing the dependence of exchange activity on external (ex) carnitine or internal (in) carnitine. Concentrations of the countersubstrate were as follows: A, 0.05 mM (open circles), 0.1 mM (filled circles), 0.2 mM (open triangles), 0.5 mM (filled triangles) external carnitine; B, 2.0 mM (open circles), 5.0 mM (filled circles), 10.0 mM (open triangles), 20.0 mM (filled triangles) internal carnitine. C, D, slope and intercept replots of primary plots A and B, respectively. These data are representative for six independent experiments.

One has to stress that although the half-saturation constants for the external side of the membrane were found to be very similar here (the carrier from rat brain) and by Indiveri et al. 1994 (the carrier from rat liver), the final conclusion as to the transport mechanism was different. Whilst the ping-pong mechanism has been postulated for liver (Indiveri et al. 1994), the sequential one is reported in the present paper.

This substantial difference, as well as the altered substrate specificity reported earlier (Kamińska et al. 1993), might be a consequence of the presence of different proteins expressed in these tissues. Such a case was already reported for some other inner mitochondrial membrane carriers. For instance, it is well established that the ADP/ATP translocase exists in several isoforms, which are expressed differently in

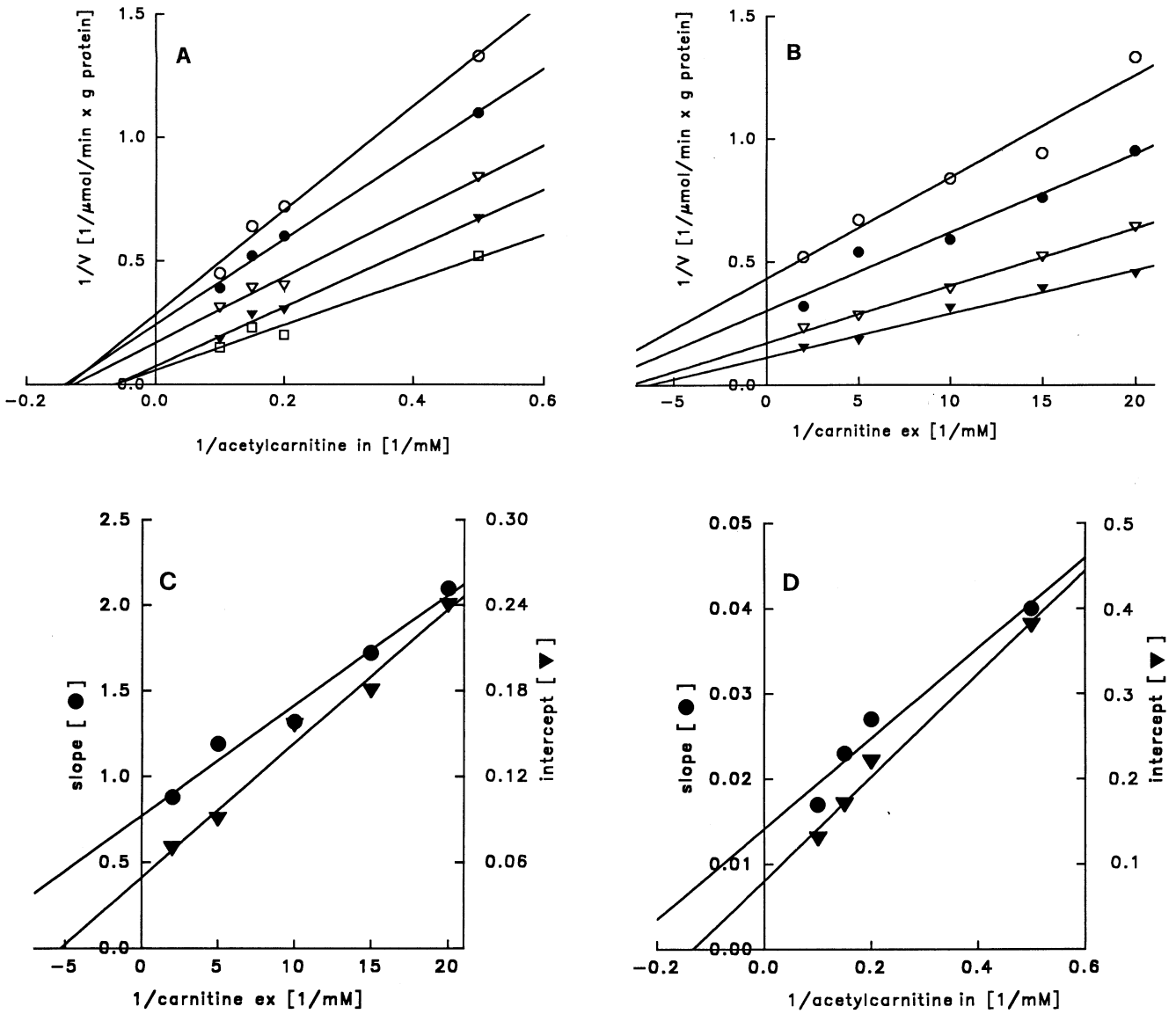

Fig. 5. Two-substrate analysis of the carnitine/acetylcarnitine heteroexchange reaction mediated by the reconstituted carnitine carrier. The transport was measured as accumulation of radioactive carnitine after 5 min as described in Methods. A, B, Lineweaver-Burk plots showing the dependence of exchange activity on external (ex) carnitine or internal (in) acetylcarnitine. The concentrations of the countersubstrate were as follows: A, 0.05 mM (open circles), 0.07 mM (filled circles), 0.1 mM (open triangles), 0.2 mM (filled triangles), 0.5 mM (open squares) external carnitine; B, 2.0 mM (open circles), 5.0 (filled circles), 7.0 mM (open triangles), 10.0 mM (filled triangles) internal acetylcarnitine. C, D, slope and intercept replots of primary plots A and B, respectively. These data are representative for four independent experiments.

various tissues (Walker and Runswick 1993), and different forms of mature phosphate carrier are differently expressed in heart and liver (Dolce et al. 1994). The observed differences in substrate specificities between carnitine carrier isolated from liver and brain could be connected with different metabolic function of this protein in these organs. It has been postulated that in liver the major function of the carnitine carrier is the transfer of acyl derivatives of carnitine into mitochondria, while in brain the carnitine carrier could play the main role in transport of acetyl groups in the opposite direction

(from mitochondria to cytoplasm), delivering acetyl moieties for syntesis of acetylcholine (Dolezal and Tucek 1981).

Taking into account the observed differences in the kinetic mechanism and substrate specificity, one could also postulate that different isoforms of carnitine carrier are present in liver and brain.

ABBREVIATIONS

DTE	dithioerytritol
HTP	hydroxyapatite
NEM	N-ethylmaleimide
SDS	sodium dodecyl sulfate

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